



Calcium and potassium content in beef: Influences on tenderness and associations with molecular markers in Nellore cattle

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ABSTRACT

Calcium (Ca) and potassium (K) are essential nutrients in animal nutrition. Furthermore, the Ca content can influence meat tenderness because it is needed by the proteolytic system of calpains and calpastatins, major factors in postmortem tenderization of skeletal muscles. K content, which is needed for muscle contraction, can also affect meat tenderness. This study showed that K positively affects the Warner–Bratzler shear force (WBSF), measured at 14 days of meat aging, which means that higher levels of K are related to lower meat tenderness. Additionally, a significant effect ($P \leq 0.015$) of a SNP in the calcium-activated neutral protease 1 (*CAPN1*) gene on Ca content was observed. Metal content in beef can affect not only nutritional values but also meat quality traits. Part of this effect may be related to variation in specific genes.

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1. Introduction

Beef is an important part of the diet in most countries. Besides the relevance of metal content in meat for human nutrition, the investigation of the effects of metal levels on meat quality is also important. Brazil is the main global producer of beef and Nellore is the main cattle breed in Brazil. Variation in tenderness is a major concern for the meat industry (Veiseth, Shackelford, Wheeler, & Koohmaraie, 2004) and Zebu cattle produces less tender meat compared to European breeds.

Metals such as calcium (Ca) and potassium (K) can affect meat tenderness through their function in the cells. Research has shown that neutral proteases dependent on calcium ions, calpains are associated with postmortem protein degradation of skeletal muscle (Geesink & Koohmaraie, 1999). The calcium-activated neutral protease 1 gene (*CAPN1*; Gene ID: 281661) codifies the μ -calpain enzyme. Calpastatin (*CAST*; Gene ID: 281039), an enzyme that inhibits the action of *CAPN1*

and is primarily responsible for the regulation of postmortem proteolytic activity (Koohmaraie, 1996). The increase in postmortem activity of *CAST* is associated with reduced beef tenderness (Pringle, Williams, Lamb, Johnson, & West, 1997). Studies have shown an association between polymorphisms in the *CAPN* and *CAST* genes and meat tenderness in different cattle populations (Barendse, 2002; Casas et al., 2006; Corva et al., 2007; Page et al., 2002; Schenkel et al., 2006).

The potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11*, Gene Bank ID: 532060) is involved in the insulin secretion pathway (Alekseev et al., 2010). It is located on bovine chromosome 15, close to a quantitative trait locus (QTL) for meat tenderness (Rexroad III et al., 2001). This gene encodes a protein which increases the flow of K into cells where it takes part in the establishment of electrical potential in the cell membrane. K is necessary for muscle contraction and nerve impulses, and along with sodium, it helps maintain the proper balance of fluids in the cells (Knochel & Schlein, 1972). A recent study (Tizioto et al., submitted for publication) found that a single nucleotide polymorphism (SNP) in the *KCNJ11* gene is related to meat tenderness in the same population used in this study. Recent results indicate the importance of genomic variation in the mineral content of different tissues (Morris et al., 2013).

In this study, the association between Ca and K content and meat tenderness was analyzed as well as the effects of SNPs in the *CAPN1*,

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CAST and *KCNJ11* genes on Ca and K contents in a population of Nellore cattle.

2. Materials and methods

2.1. Animals and phenotypic data

Nellore steers ($n = 286$), offspring of 32 sires chosen to represent the main breeding lineages in Brazil, were used to obtain genotypic and phenotypic data. The half-sib families were produced by artificial insemination in commercial and purebred Nellore dams. The animals were raised and finished in feedlots at the experimental unit of the Embrapa Cattle – Southeast research unit at São Carlos, São Paulo, Brazil. The diet of the animals was previously described (Tizioto et al., 2012). The animals were slaughtered when they reached 5 mm of back fat thickness determined by ultrasound imaging.

After slaughter, 2.5 cm thick steaks from a cross section of the *longissimus dorsi* muscle, between the 12th and 13th ribs, were collected. These were identified, vacuum-packed and analyzed for meat tenderness by Warner–Bratzler shear force (WBSF) measurements. The WBSF was measured in a TA XT2i texture analyzer coupled to a 1.016 mm Warner–Bratzler probe. The analyses of WBSF were done at different aging times: about 24 h after slaughter (WBSF0), after seven days (WBSF7) and 14 days (WBSF14). Samples were aged at 2 °C in a cold chamber manufactured by McQuay–Heatcraft do Brasil Ltda. (São José dos Campos, São Paulo, Brazil).

2.2. Sample preparation for chemical elements determination

Analytical grade reagents and Milli-Q water (Millipore, Billerica, MA, USA) were employed. All stock standard solutions used to prepare the multi-element standard solution were certified with plasma grade and high purity materials from SpecSol, (Jacareí, SP, Brazil). Working standard solutions were prepared daily by diluting appropriate aliquots of the stock solution in ultrapure water.

A closed-vessel microwave digestion system (Ethos-1600, Milestone-MLS, Sorisole, Italy) equipped with optic fiber temperature and pressure sensors was used for sample digestion. Sample masses of 100 mg of beef samples were digested with microwave assistance using 2 mL of sub-boiled concentrated HNO_3 , 2 mL of H_2O_2 (30% w/w) and 6.0 mL of ultrapure water in closed vessels made of perfluoroalkoxy copolymer resin (PFA). The heating program was in three steps: (1) heating ramp of 10 min with maximum temperature of 120 °C (1300 W); (2) second heating ramp of 15 min with maximum temperature of 170 °C (1500 W); (3) temperature at 170 °C during 35 min.

After digestion, the samples and blank solutions were transferred to 10.0 mL volumetric flasks and made to volume with deionized water. The metal concentrations were determined by a Vista Pro-CCD ICP-OES spectrometer (Varian, Mulgrave, Australia). The wavelengths were chosen according to the least spectral interference and the highest intensity emission for each element. The optimized parameters used in the ICP-OES are described in Table S1. A linear calibration was calculated with up to five points, which was prepared with standard analytical solutions.

The accuracy of the proposed method was evaluated by analyzing the certified reference material Bovine Liver 1557b and Bovine Muscle 8414 from the National Institute of Standards and Technology (NIST Gaithersburg, MD, USA). To assess possible contamination during sample preparation, blank samples of ultrapure water were prepared using the same procedure as for the beef samples.

2.3. DNA extraction

Straws of frozen semen obtained from Brazilian artificial insemination centers were used to extract DNA from bulls using the standard phenol-chloroform method (Sambrook, Fritsch, & Maniatis, 1990). For the steers, 5 mL blood samples were used and DNA extractions were

performed by a salting-out method as described in Tizioto et al. (2012). DNA concentration was measured by spectrophotometry, and the quality was verified by the 260/280 ratio, followed by inspection of integrity through agarose gel electrophoresis.

2.4. SNPs genotyping

For the *CAPN1* gene, the synonymous SNP c.3379G > A (rs17872099), located in exon 5, was genotyped in 133 animals. For the *CAST* gene, genotypes for 178 animals were determined for SNP c.2959A > G (AF159246), located in the 3'UTR region (Barendse, 2002).

Two SNPs in *KCNJ11* were genotyped in 286 animals, one (c.1526C > T; NCBI_ss#537718973) a synonymous mutation located in the coding region and the other (c.2342T > C; NCBI_ss#537718995) located in the 3'UTR region. The genotypes for all SNPs were determined by real-time PCR, using TaqMan™ assays (Applied Biosystems, Foster City, CA, USA) (Table S2).

2.5. Statistical analysis

The content of Ca and K given in mg g^{-1} were transformed into $\log\text{Ca}$ and $\log\text{K}$ to achieve a normal distribution.

A mixed model was used with fixed effects of contemporary groups (CGs) and age of the animal at measurement (linear effect), pH and metal content as covariates, and sire as the random effect. The CG included the effects of birthplace, breeding season, and slaughter date.

Analyses were performed by restricted maximum likelihood using the PROC MIXED procedure in the Statistical Analysis System (SAS) (SAS Institute Inc., 2000) using model 1:

$$Y_{ijklm} = \mu + CG_i + S_j + b_1(A_{ijk} - a) + b_2(P_{ijkl} - p) + b_3(M_{ijklm} - m) + e_{ijklm} \quad (1)$$

where:

Y_{ijklm}	observation of individual l , of age A , offspring of sire k with genotype j for the marker, belonging to contemporary group i ;
μ	overall mean;
CG_i	fixed effect of the contemporary group i ;
S_j	random effect associated with the father of animal k , $N \sim (0, \sigma_s^2)$;
b_1	linear regression coefficient associated with animal's age;
b_2	linear regression coefficient associated with pH of the samples;
b_3	regression linear coefficient associated with Ca or K content of the samples;
A_{ijk}	animal age on the date of measurement, a is the mean age of measurement;
P_{ijkl}	value for each observation of pH, p is the mean pH of the samples;
M_{ijklm}	value for each observation of metal content (Ca or K), m is the mean Ca or K of the samples;
e_{ijklm}	random error associated with each observation, assumed normally distributed with mean zero and variance σ^2 .

To evaluate the effects of markers in *CAPN1*, *CAST* and *KCNJ11* on the Ca and K content in the meat, the model described in Eq. (2) was used.

$$Y_{ijkl} = \mu + CG_i + M_j + S_k + b_1(A_{ijkl} - a) + e_{ijkl} \quad (2)$$

where:

Y_{ijklm}	observation of individual l , of age A , son sire k with genotype j for the marker, belonging to contemporary group i ;
μ	overall mean;

CG_i	fixed effect of contemporary group i ;
S_j	random effect associated with the father of animal k , $N \sim (0, \sigma_s^2)$;
M_j	fixed effect of genotype j for the marker;
S_k	random effect associated with the father of animal k , $N \sim (0, \sigma_s^2)$;
b_1	linear regression coefficient associated with animal's age;
A_{ijkl}	animal age on the date of measurement, a is the mean age of measurement;
e_{ijkl}	random error associated with each observation, assumed normally distributed with mean zero and variance σ^2 .

The probability values were not corrected for multiple testing in any analysis in this study.

3. Results and discussion

To access the accuracy of the sample preparation procedure, certified reference materials (CRMs) were evaluated. The agreement between the values obtained for the experimental samples and the CRM values indicates effective recovery of analytes after digestion and accurate detection. The certified recoveries for bovine muscle were $98.3 \pm 4.5\%$ (for Ca 396.847 nm) and $112.8 \pm 1.7\%$ (for K 769.897 nm) and the certified recoveries for bovine liver were $100.6 \pm 7.3\%$ (for Ca 396.847 nm) and $113.8 \pm 1.9\%$ (for K 769.897 nm).

The average, maximum and minimum concentrations found for Ca 396.847 in the 281 Nellore animals were 164.6, 984.1 and 49.7 mg kg^{-1} and for K 769.897 these values were 1211.2, 2301.9 and 262.3 mg kg^{-1} , respectively. Beef has all the metals considered to be important in human nutrition. Studies have shown that the content of Ca and K in skeletal muscle displays great variation (Garmyn et al., 2011; Morris et al., 2013). The samples presented a similar amount of Ca compared to other studies of commercial beef samples, in which the average was $152 \pm 15 \text{ mg kg}^{-1}$ (Matos et al., 2009). The mineral levels in meat can be affected by environmental effects such as birthplace, diet, age and breed. In the present analysis it was demonstrated that the CG significantly influences the Ca and K in the Nellore breed (Table S2), suggesting that one or more of the factors included under this classification (birthplace, breeding season and slaughter date) affect the content of both minerals.

The frequencies observed for the SNPs in this study and the number of animals genotyped for each SNP are presented in Table 1. The test of SNP genotype influence on meat Ca and K levels showed a significant effect ($P \leq 0.01$) of the *CAPN1* gene SNP c.2959A > G on calcium content. The least square means of logCa for *CAPN1* genotypes were: AA: 5.08 ± 0.14 ; GA: 5.10 ± 0.17 and GG: $4.99 \pm 0.04 \text{ mg kg}^{-1}$, with the genotypes AA and GA being significantly different from the GG genotype.

When analyzing the relationship between metal contents and WBSF, a weak negative correlation with WBSF measures was found in a preliminary Pearson correlation analyses (data not shown) for both logCa and logK, as also reported by Garmyn et al. (2011). However, when

including logCa and logK contents as covariates in statistical model 1, which also included other potential factors that influence the WBSF, only K content had a significant but positive effect on the WBSF14 ($P \leq 0.0475$). Unlike the Pearson correlation results, the positive effect of logK on WBSF14 (Table 2) was estimated after correction of the WBSF values for the other effects included in the model. No significant effect of Ca on either WBSF measure was observed. The estimated effect of K on WBSF was $2.98 \text{ kgf/mg kg}^{-1}$.

K has long been recognized as an essential element and its deficiency can cause stunted growth (Fervenza, Tsao, & Rabkin, 2001), muscular weakness (Bilbrey, Herbin, Carter, & Knoche, 1973), and decreased feed intake (Campbell & Roberts, 1965), among other effects. It is necessary for muscle contraction and nerve impulses, and along with sodium, it helps maintain the proper balance of fluids in human cells (Knoche & Schleim, 1972). Deficiency of K causes muscular weakness, a condition that can result in more tender meat after slaughter. Considering this, the results of a positive effect of K content on WBSF14 measures seem logical, since it means that more K in meat is related to less tender meat. A study conducted in humans showed that the weakness of periodic paralysis might be due to atypical absorption of K by muscles, which causes an increase in the intracellular to extracellular concentration ratio of this ion. This result in hyperpolarization of the muscle membrane, in turn related to reduction in muscle responsiveness to nerve stimulation, propagation of excitation and contraction (Grob, Johns, & Liljestrand, 1957).

Studies have shown that changes in osmotic pressure do not stop at 24 h postmortem, but are active throughout storage (Veiseth et al., 2004). Considering these known effects of K and the results shown here, providing a correct diet and assuring that animals have an adequate absorption and utilization of K, besides being important for animal growth and muscular force, may improve meat tenderness. However, the effect of K on meat aging is unknown and further studies are needed to elucidate it.

The *KCNJ11* gene encodes a protein which increases the flow of K into cells, where it takes part in the establishment of electrical potential in the cell membrane, an essential mechanism for muscle contraction. A study has shown that disruption of muscle-specific KATP channel function resulted in a higher cost of physical activity (Aleksiev et al., 2010). The role of this gene in muscle contraction may underlie an effect of *KCNJ11* genotypes on meat tenderness, as pointed out by Tizioto et al. (submitted for publication). The higher level of K in cells can increase the levels of muscle contraction, which should reduce the tenderness of meat. No significant association between the SNPs in the *KCNJ11* evaluated in this study and total K content in the meat could be observed. In this analysis, it was not possible to distinguish the K content in the intracellular and extracellular spaces, so a possible effect on the regulation of this flow would not be detected.

Many studies have suggested *CAPN1* and *CAST* genes as candidates to influence meat tenderness. The *CAPN1* gene encodes the protease μ -calpain, which is the major enzyme involved in degradation of muscle myofibrils, and their function has been associated with meat tenderness in the postmortem period. The μ -calpain activity is modulated by calpastatin, which is the only inhibitor and is encoded by the *CAST* gene (Koochmarie, 1996). The increase in activity of *CAST* in the postmortem period was associated with reduction of tenderness (Pringle et al., 1997). In this study, it was found that the marker c.3379G > A in the *CAPN1* gene influences the Ca content in the meat of this Nelore population, with the rare genotype associated with less Ca content. This result suggests this SNP as a marker for Ca content in meat, however, considering the low frequency of GG genotype in the sample used (Table 1), other studies with more animals are necessary to confirm this effect. The calpain system is highly sensitive to fluctuating levels of Ca ions, pH and temperature, although these three parameters change rapidly in the immediate postmortem period (Suzuki, Sorimachi, Yoshizawa, Kinbara, & Ishiura, 1995). Whether the difference in Ca content related to the polymorphism in *CAPN1* affects calpain and calpastatin

Table 1
Allelic and genotypic frequencies of *CAPN1*, *CAST* and *KCNJ11* markers.

Gene/SNP	Frequency (%)				
	Allelic		Genotypic		
<i>CAPN1</i> /c.2959A > G	A	G	AA	AG	GG
$N^a = 133$	80.33	19.67	64.34	31.97	3.69
<i>CAST</i> /c.2959A > G	A	G	AA	AG	GG
$N^a = 178$	44.66	55.34	16.85	55.62	27.53
<i>KCNJ11</i> /c.1526C > T	C	T	CC	CT	TT
$N^a = 286$	76.22	23.78	54.20	44.06	1.75
<i>KCNJ11</i> /c.2342 T > C	C	T	CC	CT	TT
$N^a = 286$	53.69	46.31	36.90	33.58	29.52

^a N = Number of animals genotyped with phenotype measures.

Table 2

Summary of analysis of variance (ANOVA) for WBSF measurements considering calcium (logCa) and potassium (logK) as covariates in the mixed models.

WBSF	N	1				2			
		Source	DF ^a	Mean square	Pr > F	Source	DF ^a	Mean square	Pr > F
0	261	CC ^b	13	13.8073	<.0001	CC ^b	13	14.4113	<.0001
		Sire	31	2.5017	0.0895	Sire	31	2.4905	0.0922
		Age ^c	1	5.1685	0.0909	Age ^c	1	4.7824	0.1037
		pH	1	8.0260	0.0355	pH	1	8.2230	0.0333
		LogCa	1	1.7911	0.3185	LogK	1	1.9570	0.297
		Residue	213	1.7915	–	Residue	213	1.7907	–
		R ² = 0.48 ^d				R ² = 0.48 ^d			
7	248	CC ^b	13	5.6806	0.0017	CC ^b	13	5.8746	0.0012
		Sire	31	2.6999	0.1651	Sire	31	2.6333	0.187
		Age ^c	1	3.9046	0.1764	Age ^c	1	3.4883	0.2005
		pH	1	0.0204	0.922	pH	1	0.0257	0.9123
		LogCa	1	0.2543	0.7295	LogK	1	1.5657	0.3906
		Residue	200	2.1213	–	Residue	200	2.1148	–
		R ² = 0.31 ^d				R ² = 0.31 ^d			
14	259	CC ^b	13	5.6842	<.0001	CC ^b	13	6.7929	<.0001
		Sire	31	1.3774	0.7026	Sire	31	1.3289	0.7274
		Age ^c	1	4.5286	0.0968	Age ^c	1	3.5925	0.1359
		pH	1	3.8986	0.1232	pH	1	3.9781	0.1167
		LogCa	1	1.2599	0.3800	LogK	1	6.3705	0.0475
		Residue	211	1.6278	–	Residue	211	1.6036	–
		R ² = 0.40 ^d				R ² = 0.41 ^d			

1 = mixed model used to evaluate the sources that influence the Warner–Bratzler shear force (WBSF) measures at 24 h (WBSF0), seven days (WBSF7) and fourteen days (WBSF14) after slaughter, including logCa as a covariate; 2 = mixed model used to evaluate the sources that influence the WBSF measures, including logK as a covariate.

^a Degree of freedom.

^b Contemporary group.

^c Animal age at slaughter.

^d R-squared.

activity, taking into account their Ca dependence, should be further investigated.

4. Conclusions

Chemical analysis of metal content in beef can be useful to investigate the effects of metal contents on meat quality related traits such as the effect of K content on tenderness at 14 days of aging observed in the present study.

Besides nutrition and other environmental factors, meat Ca content can be affected by genetic variation at *CAPN1*, which can be exploited through selection.

Further studies with larger sample sizes and different populations are necessary to validate the results in this work.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.meatsci.2013.08.001>.

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